

Embryonic Cell Lineages in the Nervous System of the Glossiphoniid Leech *Helobdella triserialis*

DAVID A. WEISBLAT, GEORGIA HARPER, GUNTHER S. STENT, AND ROY T. SAWYER

Department of Molecular Biology, University of California, Berkeley, California 94720

Received August 14, 1979; accepted in revised form October 15, 1979

The lines of descent of cells of the nervous system of the leech *Helobdella triserialis* have been ascertained by injection of horseradish peroxidase (HRP) as a tracer into identified cells of early embryos. Such experiments show that the nervous system of the leech has several discrete embryological origins. Some of the neurons on one side of each of the segmental ganglia derive from a single cell, the ipsilateral N ectoteloblast. Other neurons derive from a different precursor cell, the ipsilateral OPQ cell that gives rise to the O, P, and Q ectoteloblasts. The positions within the ganglion of neuronal populations derived from each of these sources are relatively invariant from segment to segment and from specimen to specimen. Other nerve cord cells derive from the mesoteloblast M; of these four per segment appear to be the precursors of the muscle cells of the connective. The A, B, or C macromeres contribute cells to the supraesophageal ganglion. In preparations in which an N ectoteloblast was injected with HRP after production of its bandlet of *n* stem cells had begun, the boundary between unstained (rostral) and stained (caudal) tissues can fall within a ganglion or between ganglia. This suggests that each hemiganglion contains the descendants of more than one, and probably two, *n* stem cells.

INTRODUCTION

In 1878, C. O. Whitman, working with the glossiphoniid leech *Hemiclepsis marginata* (Annelida: Hirudinea), showed that a definite developmental fate can be assigned to each blastomere of the early embryo. Glossiphoniid leeches are suitable for such studies because their eggs are large (with a diameter of about 1 mm) and undergo stereotyped cleavages that produce an early embryo containing large, identifiable cells. These embryos can be observed, manipulated, and cultured to maturity in simple media. Moreover, the development from egg to adult is direct, without larval structures. A comprehensive review of leech embryogenesis can be found in Schleip (1936).

We have recently devised a new method for determining developmental cell lineages that extends and refines the results obtained by direct observation (Weisblat *et al.*, 1978). This method uses horseradish peroxidase (HRP), as an intracellular

tracer, injected through a micropipet into identified cells of early embryos. After HRP injection, embryonic development is allowed to progress to a later stage, at which time the distribution pattern of HRP within the embryonic tissues is visualized by histochemical staining. In this way one can determine the developmental origin of cells whose small size, envelopment by morphologically similar cells, or remote descent from an identifiable progenitor prevents determination of their lineage by direct observation. The success of the HRP method requires that three conditions be satisfied: (i) after injection of HRP, embryonic development must continue normally; (ii) the injected HRP must remain active and not be diluted too much in the developing embryo; and (iii) HRP must not pass through junctions linking embryonic cells. These conditions are met when the HRP method is applied to embryos of the small glossiphoniid leech *Helobdella triserialis*. Initial experiments demonstrated, furthermore,

that the developmental fates assigned to the identified blastomeres of the leech embryo by Whitman are largely correct (Weisblat *et al.*, 1978).

We now present a more extensive account of cell lineage in *Helobdella* embryos using the HRP tracer method. In particular, we report in greater detail the results of an analysis of the developmental origins of the components of the leech central nervous system (CNS).

MATERIALS AND METHODS

Experiments were carried out with the small (1 cm) glossiphoniid leech *Helobdella triserialis* (Em. Blanchard, 1849; *H. lineata*, Verrill, 1874). A breeding colony of this leech has been maintained in this laboratory since 1976. The colony is kept in glass aquaria filled with aerated commercial spring water at 25°C. The leeches feed on pond snails (*Physa sp.*) obtained from a commercial supplier. Under these conditions, the egg-to-egg generation time is about 6 weeks. Like all leeches, *Helobdella* is hermaphroditic; gravid individuals can be detected several days prior to egg deposition by the appearance of swollen ovisacs, visible through the midventral body wall. Such individuals are transferred to a small dish filled with spring water for collection of early embryos. A clutch may consist of as many as fifty eggs, distributed over four or five cocoons attached to the ventral surface of the parent. To secure the embryos, the parent is transferred to a dish containing *Helobdella* embryo medium (HL medium) (4.8 mM NaCl, 1.2 mM KCl, 2.0 mM MgCl₂, 5.0 mOsm Tris maleate, pH 6.6), and the cocoons are removed with forceps (Dumont No. 5). The embryos are teased from the cocoons with insect pins and washed in fresh HL medium. Embryos are transferred to a small covered dish and the medium is changed daily. They can be cultured from uncleaved egg to adulthood, first in HL medium and, after hatching from the vitelline membrane, in spring water. To enhance survival of injected embryos 50 µg/

ml gentamycin sulfate (Schering) is added to the medium. The parent is usually unharmed by the removal of its cocoons; individual *Helobdella* leeches can produce several broods of offspring.

The procedures for HRP injection and histochemical processing of the embryos were basically those described previously (Weisblat *et al.*, 1978). For injection, the embryo was placed on the tip of a hollow plastic tube, oriented with the target cell facing upward, and then immobilized by gentle suction through the tube. A highly purified form of HRP (Type IX, Sigma) was used. No attempt was made to quantify the amount of HRP injected per cell. Excessive injection usually causes swelling, and such cells fail to divide. Some injected embryos may leak yolky cytoplasm following HRP injection and yet develop normally. A successfully injected teloblast may stain weakly, even though its descendant stem cells stain strongly. Although for many purposes it would be desirable to let the HRP-injected embryos develop to adulthood before fixing and staining for HRP, several factors mitigate against the success of such experiments. Among these factors are dilution, and possible breakdown and excretion, of the injected HRP, and a higher posthatching mortality of injected embryos.

Fixation was carried out with 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 6.6 for 10–15 hr at 4°C. Serial thick sections (1 µm) of Epon-embedded specimens were counterstained with toluidine blue, and photographed under phase contrast optics.

Germinal bands were stained by transferring live embryos from HL medium to Mayer's hematoxylin. Optimal staining was reached within 2–20 min, with the longer times being required for embryos at less advanced stages. The embryos were then rinsed in HL medium and photographed. To observe glial cell bodies in the central nervous system of adult *Helobdella*, specimens were fixed in Carnoy's fixative,

embedded in paraffin, sectioned, and stained with hematoxylin-phloxine safran (Luna, 1968).

RESULTS

Staging of the Development of the Embryo

In this report we describe the early development of *Helobdella triserialis* in terms of the nomenclature and staging system used by Fernandez (1980) for embryos of the glossiphoniid leech *Theromyzon rude*. This system, as adapted to *H. triserialis*, is summarized briefly in the following

material and illustrated schematically in Fig. 1. The criteria for each stage are italicized. Times given are approximate and pertain to embryos at 25°C.

Stage 1. Uncleaved egg. *From the time of egg laying to the onset of first cleavage* (0–4.5 hr). The egg of *Helobdella* is about 500 μm in diameter and is encased in a transparent vitelline membrane. Most of the egg cytoplasm consists of pink yolk. Before the first cleavage the egg elongates and regions of clear cytoplasm, or polar plasm, appear in the yolk. Polar bodies become visible

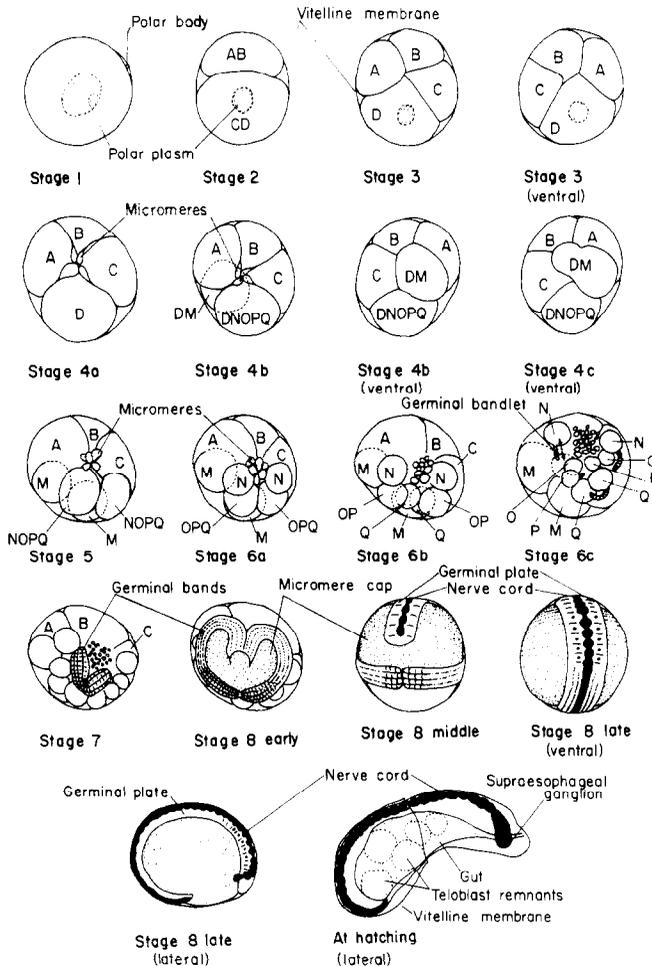


FIG. 1. Schematic representation of the first eight stages and of a hatching embryo in the development of *Helobdella triserialis*. Unless designated otherwise, all drawings are dorsal views, with left and right sides of the embryo lying to the left and right of the drawing, respectively. The two lateral views are shown venter upward, with the head lying to the right. All stages are drawn to the same scale; the diameter of the uncleaved egg (stage 1) is about 500 μm. In the drawing of stage 6c two alternative positions of cell N1 are shown, one in solid and one in dotted outline, connected by a double-headed arrow.

during this stage.

Stage 2. Two cells. *From the onset of first cleavage to the onset of second cleavage* (4.5–6.5 hr). The egg cleaves, resulting in a smaller cell AB and a larger cell CD that receives the bulk of the polar plasm.

Stage 3. Four cells. *From the onset of cleavage of cell CD to the onset of micromere cleavage* (6.5–8 hr). Cell CD cleaves to produce cells C and D; then cell AB cleaves to produce cells A and B. The bulk of the polar plasm goes to cell D. Each of the four cells can be identified unambiguously by the pattern of the cleavage furrows, as shown in Fig. 1.

Stage 4. *From the onset of micromere formation to the onset of cleavage of cell DNOPQ* (8–14 hr). This stage can be further subdivided as follows:

Stage 4a. Micromere quartet. *From the onset of micromere formation to the onset of cleavage of the D macromere to form cells DNOPQ and DM*. At the dorsal junction of the four cleavage furrows, each of the four cells cleaves to produce a macromere and a much smaller micromere, beginning with cell D. Under the present usage, the macromere retains the capital letter designation of the parent cell, and the micromere is assigned the corresponding lower case letter. Additional rounds of micromere formation directly from the macromeres have been reported for other glos-siphoniid embryos (Schleip, 1936). It has not yet been established whether such additional rounds occur in *Helobdella*.

Stage 4b. Macromere quintet. *From the onset of D macromere cleavage to the onset of cleavage of cell DM*. The D macromere cleaves to produce cells DNOPQ and DM. Since Whitman, cell DNOPQ is regarded as the source of ectoderm, and DM as the source of mesoderm. [Under the traditional annelid spiral cleavage nomenclature, these daughter cells of cell D are designated as *xd* and *yD*, respectively, where *x* and *y* are arabic numerals that designate the number of intervening cleavages since formation of

cell D (Anderson, 1973); more recently they have been designated as D1 and D2 (Weisblat *et al.*, 1978). We prefer the present designation of these cells as DNOPQ and DM because it is suggestive of their fate and avoids the ambiguity arising from interspecies variations in *x* and *y*.]

Stage 4c. Mesoteloblast formation. *From the onset of cleavage of cell DM to the onset of cleavage of cell DNOPQ*. Cell DM cleaves to yield the right and left mesoteloblasts *Mr* and *Ml*. This cleavage is not readily apparent, because one of the daughter cells, the right mesoteloblast *Mr*, comes to lie beneath the surface of the embryo, and the other, the left mesoteloblast *Ml*, occupies nearly the same position as the parent cell DM. Nevertheless, the cleavage is manifest first by the constriction of cell DM and second by the smaller size of its daughter mesoteloblast *Ml*.

Stage 5. Ectoteloblast precursor. *From the onset of cleavage of cell DNOPQ to the onset of cleavage of the NOPQ cell pair* (14–17 hr). Cell DNOPQ cleaves symmetrically to yield the right and left ectoteloblast precursors *NOPQr* and *NOPQl*.

Stage 6. Teloblast completion. *From the onset of cleavage of the NOPQ cell pair to the completion of cleavage of the OP cell pair* (17–30 hr). During this stage, bilaterally homologous cells cleave synchronously. This stage can be subdivided as follows:

Stage 6a. N Teloblast formation. *From the onset of cleavage of cell NOPQ to the onset of cleavage of cell OPQ*. Cell NOPQ cleaves, yielding the smaller ectoteloblast N and the larger cell OPQ.

Stage 6b. Q teloblast formation. *From the onset of cleavage of cell OPQ to the onset of cleavage of cell OP*. Cell OPQ cleaves, yielding the smaller ectoteloblast Q and the larger cell OP. This cleavage is difficult to observe directly because the Q daughter cell pair is partly hidden by the overlying OP cell pair. It can, however, be detected by the conversion of the larger,

contiguous OPQ cell pair into the smaller, separated OP cell pair.

Stage 6c. O and P teloblast formation. *From the onset to the completion of cleavage of cell OP.* Cell OP cleaves yielding the final ectoteloblasts O and P.

Thus at the conclusion of stage 6, one bilateral pair of mesoteloblasts (*Ml* and *Mr*) and four bilateral pairs of ectoteloblasts (*Nl* and *Nr*, *Ol* and *Or*, *Pl* and *Pr*, *Ql* and *Qr*) have been formed. Of these the M teloblasts are the largest and the O and P teloblasts are the smallest, with the N and Q teloblasts being of an equal, intermediate size. [Our teloblast designations of M, N, O, P, and Q correspond to the designations My, N, M¹, M², and M³ of Schleip (1936).] The members of each cell pair are not symmetrically placed with respect to the midline, with the M teloblast pair showing the highest degree of bilateral asymmetry. Moreover, there occurs some variation in cell position from embryo to embryo; for instance, although cell *Nl* is usually separated from the cells *Ol* and *Pl* by cell *Ml*, in some specimens *Nl* lies immediately adjacent to *Ol* and *Pl*. In such cases, however, *Nl* can still be distinguished from *Ol* and *Pl* by its larger size and by its lateral position. Further cleavages of ectoteloblasts or their

precursors, resulting in the formation of additional micromeres during stages 5 and 6, have been reported for other glossiphoniid embryos (Müller, 1932; Fernandez, 1980). We have not yet been able to observe such cleavages reliably in our preparations.

Stage 7. Germinal band formation. *From the completion of cleavage of the OP cell pair to the onset of coalescence of right and left germinal bands* (30–78 hr). Each teloblast initiates a series of unequal divisions that produces a column of micromeres, one cell wide, which we designate *stem cells*. Each column of stem cells is called a *germinal bandlet*. In accord with the convention indicated above, the stem cells are designated by a lower case letter corresponding to their teloblast of origin. The initiation of germinal bandlet formation occurs in the same sequence as that in which the teloblasts arise. Thus formation of the m bandlet is already under way at stage 6. On each side the germinal bandlets merge to form the *germinal band*, as shown in Figs. 2a and b. The left and right germinal bands then move rostrally on the surface of the embryo as more stem cells are added to each bandlet. In the germinal bands, the m bandlet arising from the M mesoteloblast lies under the n, o, p, and q

FIG. 2. Photomicrographs of HRP-injected *Helobdella* embryos, viewed in wholemount, from the dorsal aspect in (a) and (b) [with future head at top], from the ventral aspect in (c), (d), and (f) [with head at left in (c) and (d), and on top in (f)], and from the lateral aspect in (e) and (g) [with head in center in (e) and on right in (g)]. Scale bar: 250 μ m. (a) Cell DNOPQ was injected at stage 4b. Fixation and staining occurred at late stage 7. The n, o, p, and q germinal bandlets and bands are bilaterally stained. The ectoteloblasts are lightly stained. This result demonstrates that cell DNOPQ is the progenitor of left and right ectoteloblasts. (b) Cell *Nr* was injected at early stage 7. Fixation and staining occurred at early stage 8. The right n germinal bandlet is stained. The *Nr* teloblast is lightly stained. The initially rearward projection of the n bandlet and its later frontward reversal at the junction with the other bandlets are manifest. This result demonstrates that a teloblast gives rise to a column of stem cells. (c) Cell *Nl* was injected at stage 6a. Fixation and staining occurred 6 days later. All left (apparent right) hemiganglia of the ventral nerve cord are partially stained. (d) Cell OPQl was injected at stage 6a. Fixation and staining occurred 6 days later. Left (apparent right) body wall is stained, as are some sectors of left hemiganglia. These stained sectors appear to correspond to those sectors that are unstained in the embryo shown in (c). (e) Right: lateral view of the embryo shown in (c) (cell *Nl* injected). Left: lateral view of an embryo similar to that shown in (d) (cell OPQr injected). Neither embryo shows staining in the supraesophageal ganglion. (f) Cell *Ml* was injected at stage 4c. Fixation and staining occurred 6 days later. The left (apparent right) germinal plate is broadly stained. (g) Injection of an A, B, or C macromere at stage 3. (The exact identity of the injected macromere was not determined.) Fixation and staining occurred 6 days later. Parts of the supraesophageal ganglion and body wall are stained. The large area of intense staining in the center of the embryo derives from the incorporation of the injected macromere into the primordial gut.

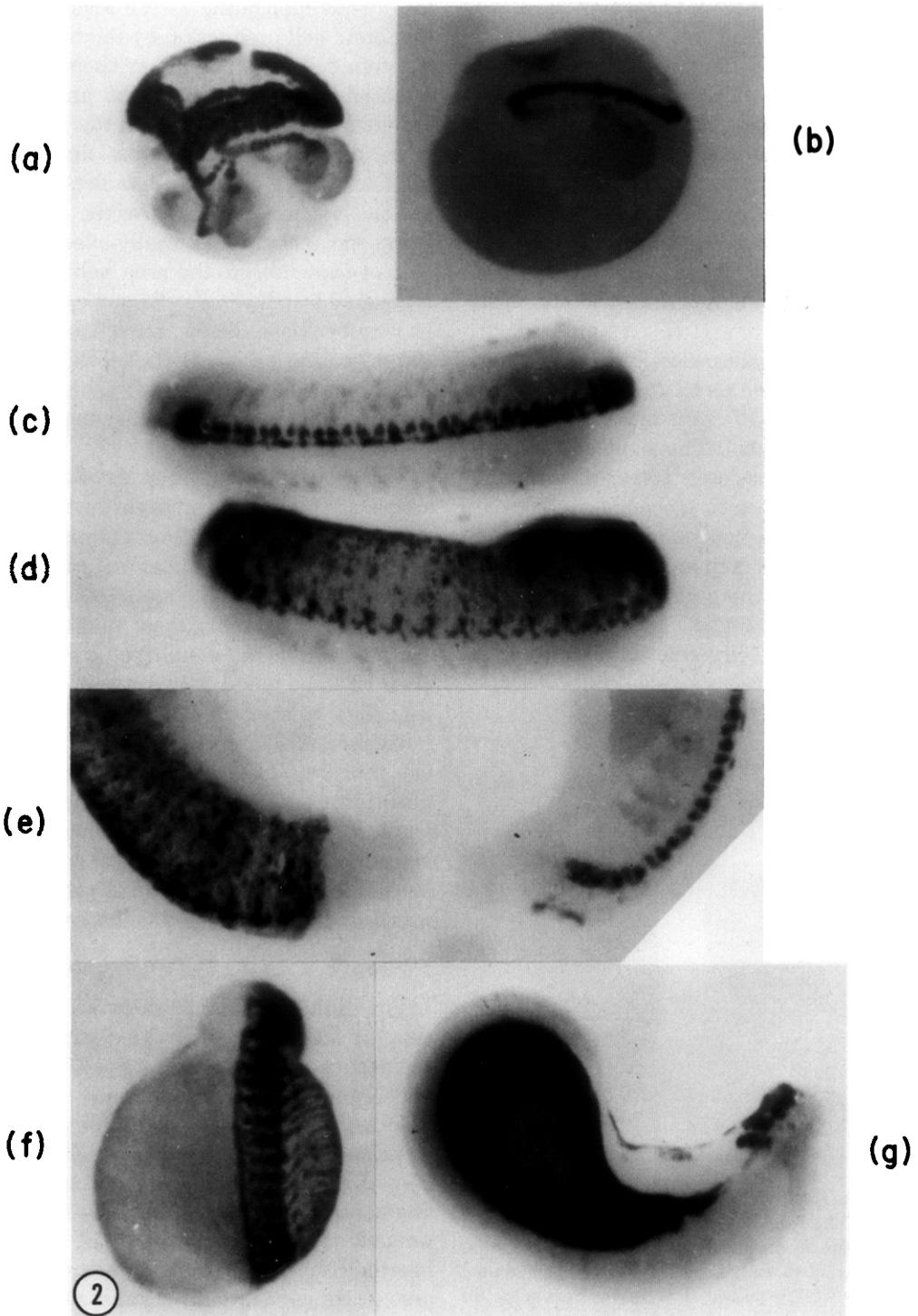


FIG. 2.

bandlets arising from the N, O, P, and Q ectoteloblasts. The ectodermal bandlets lie in the order of their alphabetic designations, with the q bandlet pair lying most medially. The germinal bands grow in crescent shape around the *micromere cap* (Mann, 1962). This growth entails a circumferential migration over the surface of the embryo, attended by an expansion of the micromere cap. Right and left germinal bands meet at the future rostral end of the animal.

Stage 8. Coalescence of the germinal bands. *From the onset to the termination of coalescence of right and left germinal bands* (78–122 hr). The right and left germinal bands coalesce zipper-like at their lateral edges in a rostrocaudal progression along the future ventral midline to form the *germinal plate*. Hematoxylin stain is useful for visualizing the germinal bands and the germinal plate (Fig. 3). The germinal bands continue their circumferential migration,

meanwhile elongating as the result of further stem cell production by teloblasts and by stem cell divisions. After their circumferential migration, the right and left n bandlets lie most medially in the germinal plate, directly apposed across the ventral midline. The o, p, and q bandlets lie progressively more laterally. As the germinal bands move circumferentially over the surface of the embryo, the area behind them is covered by a layer of cells, some of which, as is to be shown below, are left behind by the migrating bands. Even before the germinal bands have completely coalesced the embryo begins peristaltic movements along its longitudinal axis.

The definition of later developmental stages of *Helobdella* embryos awaits more detailed observations. The germinal plate expands in a rostrocaudal progression to cover the surface of the embryo, until right and left edges finally meet on the dorsal midline. *Helobdella* embryos hatch from their vitelline membrane at about the sixth day. The bulk of the yolk of the five teloblast pairs is not passed on to their stem cell progeny. It is eventually enveloped by endoderm and, together with the yolk of the A, B, and C macromeres, comprises the content of the gut of the young leech. According to Whitman (1878), the endoderm arises from the A, B, and C macromeres.

Development of the Nervous System

The *Helobdella* embryo shows the first signs of segmentation at the rostral end of the germinal plate during stage 8, with the appearance of longitudinally iterated blocks of tissue. In register with each block of tissue there develops a primordial segmental ganglion of the ventral nerve cord. Segmentation and ganglionic development progress rostrocaudally. By the time of hatching the rostral ganglia of the embryonic nerve cord already possess many features of the adult ganglia.

The adult nervous system of *Helobdella* is similar to that of other leeches, such as *Hirudo medicinalis* (Nicholls and Van Es-

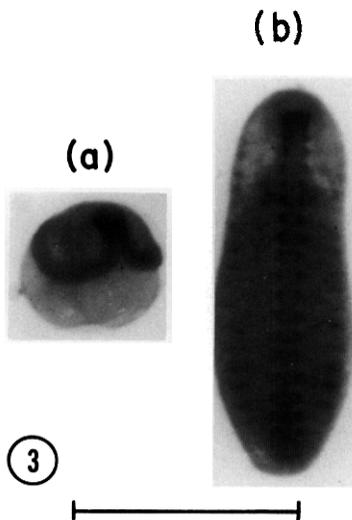


FIG. 3. Hematoxylin-stained embryos. (a) Early stage 8. The germinal bands are heavily stained and have begun to coalesce at the future head (at top). The micromere cap, lying dorsal to the germinal bands, is weakly stained. The yolk is unstained. (b) Shortly after hatching, viewed from the ventral aspect (head at top). The ganglia of the abdominal sector of the nerve cord have begun to move apart. The ganglia are heavily stained except for their central nerve tract. Scale bar: 500 μ m.

sen, 1974). The front end of the nerve cord is formed by the supraesophageal ganglion, which is linked to the subesophageal ganglion by a pair of circumesophageal connectives. The subesophageal ganglion, in turn, is followed by 21 segmental ganglia linked by the nerve tracts of the interganglionic connectives. The rear end of the nerve cord is formed by the caudal ganglion. A pair of segmental nerves exits from the left and from the right side of each segmental ganglion. Each segmental ganglion contains on the order of 380 neurons (Macagno, 1980), distributed over six distinct *cell packets*, separated by sheets of connective tissue. There are two *lateral* packets on each side, one pair anterior and the other pair posterior, extending over both the ventral and the dorsal aspects of the ganglion, and two *ventromedial* packets, one anterior and the other posterior. The cell packets constitute a cortex of the ganglion. Within the ganglion lies a neuropil of fine processes emanating from neuronal cell bodies both in its own cell packets and in other ganglia of the nerve cord. The roughly bilaterally symmetric right and left halves of the ganglion will be referred to here as *hemiganglia*. In addition to neuronal cell bodies, each hemiganglion contains also a small number of glial cells, as do the interganglionic connectives. *Hirudo* has one bilateral pair of giant glial cell bodies per segment located midway between the segmental ganglia in the core of the intersegmental connectives. A second pair is located on the ventral aspect of the neuropil of the segmental ganglion. In addition, there is one giant glial cell in each of the six segmental cell packets (Coggeshall and Fawcett, 1964). The disposition, and possibly number, of glial cells in the interganglionic connectives of *Helobdella* differs from that in *Hirudo*, however. *Helobdella* has two bilateral pairs of giant glial cell nuclei in its interganglionic connectives, of which one pair is located in the core at each end of these connectives (Figs. 4a and b). On the basis of serial transverse sections viewed under the light microscope it seems

likely that these two pairs of nuclei belong to two different pairs of glial cells, rather than to one binucleate cell pair. Just as *Hirudo*, *Helobdella* also has a pair of nerve tract glial cell bodies on the ventral aspect of the ganglionic neuropil (Fig. 4c). Furthermore, as is the case in *Hirudo* (Coggeshall and Fawcett, 1964), the sheath of the interganglionic connective of *Helobdella* contains longitudinally oriented muscle cells (Fig. 4b).

At the time of hatching the rostral segmental ganglia of the embryonic nerve cord have moved apart; neuropil, connectives, and segmental nerves are present; and the number of nerve cell bodies in the ganglion of the embryo is about the same as in that of the adult. But neither the nerve cell packets nor their glial cells are as yet clearly discernible, and in the connectives giant glial cell bodies are still missing from the position at which they are found in the adult.

Whitman (1878; 1887), and later workers (e.g., Bergh, 1891) inferred from direct observations that the ventral nerve cord derives from the N teloblast pair. But as for the supraesophageal ganglion, Whitman (1887) asserted that it has an origin other than the germinal bands. In contrast to Whitman, Müller (1932) claimed that the supraesophageal ganglion does arise from the germinal bands. It is hardly surprising that conclusions regarding the developmental origin of tissues of late leech embryos based on direct observations are controversial; after stage 6, it is difficult to follow the fate of stem cells because they and their descendant cells are small, numerous, and often hidden from view. Therefore we reexamined the question of the developmental origin of the leech CNS by means of the HRP tracer method.

Origin of the Segmental Ganglia

In order to test the traditional view that the ventral nerve cord arises from the N teloblast pair, N teloblasts of stage 6a *Helobdella* embryos were injected with HRP.

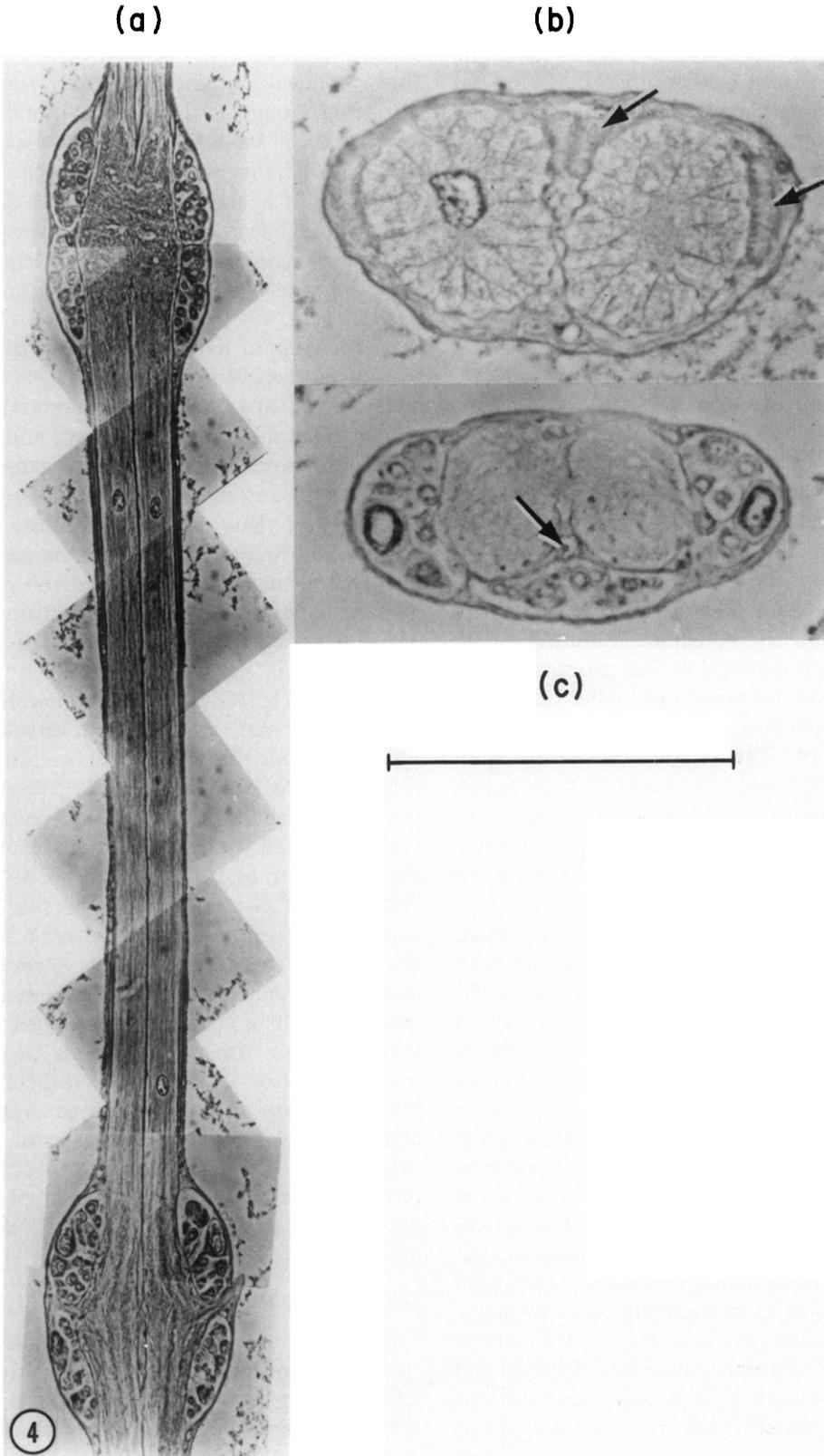


FIG. 4.

The embryos were then allowed to develop for 6 more days, after which they were fixed and stained for HRP. Wholemounts of such preparations (Fig. 2c) show that each N teloblast is indeed the progenitor of a substantial part of the ipsilateral half of the nervous system. HRP stain can be seen on the same side as the injected N teloblast in segmentally repeated structures of the appropriate size, shape, and location for hemiganglia of the ventral nerve cord. The hemiganglia are not stained uniformly; within each hemiganglion the HRP stain appears as a thin longitudinal strip next to the ventral midline and as two transverse strips extending laterally from it.

The presence of unstained tissue between the two stained transverse strips in each ganglion following HRP injection of the ipsilateral N teloblast raises the possibility that some nerve cell bodies within the hemiganglion are not derived from that cell. In addition, since the stain does not extend across the ventral midline, it follows from symmetry that any unstained cell bodies within the stained hemiganglion are not derived from the uninjected *contralateral* N teloblast.

The existence of unstained cell bodies within stained hemiganglia is confirmed by serial sections of such preparations (Fig. 5a). The two transverse strips of stain visible in wholemount correspond to anterior and posterior groups of cells in the lateral part of the ganglion. Moreover, the thin longitudinal strips of stain visible in

wholemount correspond to stained cell bodies in the ventromedial part of the ganglion. Groups of unstained cell bodies are seen to lie between the stained cells, in the central region of the ganglion.

Two additional groups of cells of the nerve cord remained unstained following HRP injection of N teloblasts. The first group consists of two readily identifiable pairs of cells at the anterior edge of the dorsal aspect of the nerve tract of each embryonic ganglion. The nature of these cells, hereafter referred to as "nerve tract" cells, will be discussed further below. The second group of unstained cells consists of the supraesophageal ganglion (Fig. 2e). It might thus be inferred that both of these groups of cells are not derived from the N teloblasts, but such negative evidence is not incontrovertible. It is possible that the unstained cells do derive from the injected teloblast, but contain a lower intracellular concentration of HRP than the stained members of the clone because they (a) are products of more generations of cell growth and division, or (b) have increased their own cell volume to a greater extent, e.g., by growth of more extensive cell processes, or (c) have developed specific metabolic or enzymatic features that engender a more rapid destruction or elimination of HRP. Moreover, in the case of the supraesophageal ganglion the additional possibility exists that by the time the N teloblast had been injected, its first stem cells had already been produced. In that case, the su-

FIG. 4. Hematoxylin-phloxine-safran stained sections of a paraffin-embedded adult specimen of *Helobdella*. (a) Horizontal section through the nerve cord at a dorsal level, showing two ganglia linked by the connective. Within the ganglia nerve cell bodies of the anterior and posterior lateral packets surrounded by the connective tissue sheet are visible. (At this dorsal level, the ventromedial cell packets cannot be seen.) Near either end of the connective, the nuclei of a pair of giant glial cells can be seen (one of these nuclei lying slightly out of the plane of the section, is only faintly visible). (b) Transverse section through the connective, showing radially oriented glial processes and, in the center of the left connective, the nucleus of one glial cell. Two pairs of longitudinally oriented muscle cells, one pair dorsomedial and the other lateral, can be seen in the sheath of the connective. The arrows point to the muscle cells of the right connective. (c) Transverse section through a ganglion. The arrow points to a glial cell body visible on the midline of the ventral edge of the neuropil, lying in the nerve tract just above the ventromedial cell packet. The second glial cell of this pair lies out of the plane of the section. [For comparison of these sections with corresponding findings in *Hirudo*, cf. Fig. 2 of Coggeshall and Fawcett (1964).] Scale bar: (a) 270 μm ; (b) 50 μm ; (c) 100 μm .

praesophageal ganglion might have derived from the eldest n stem cells that did not contain HRP. Thus in order to identify

positively the progenitors of the unstained groups of CNS cells, HRP injections of other early embryonic cells were under-

(a)

(b)

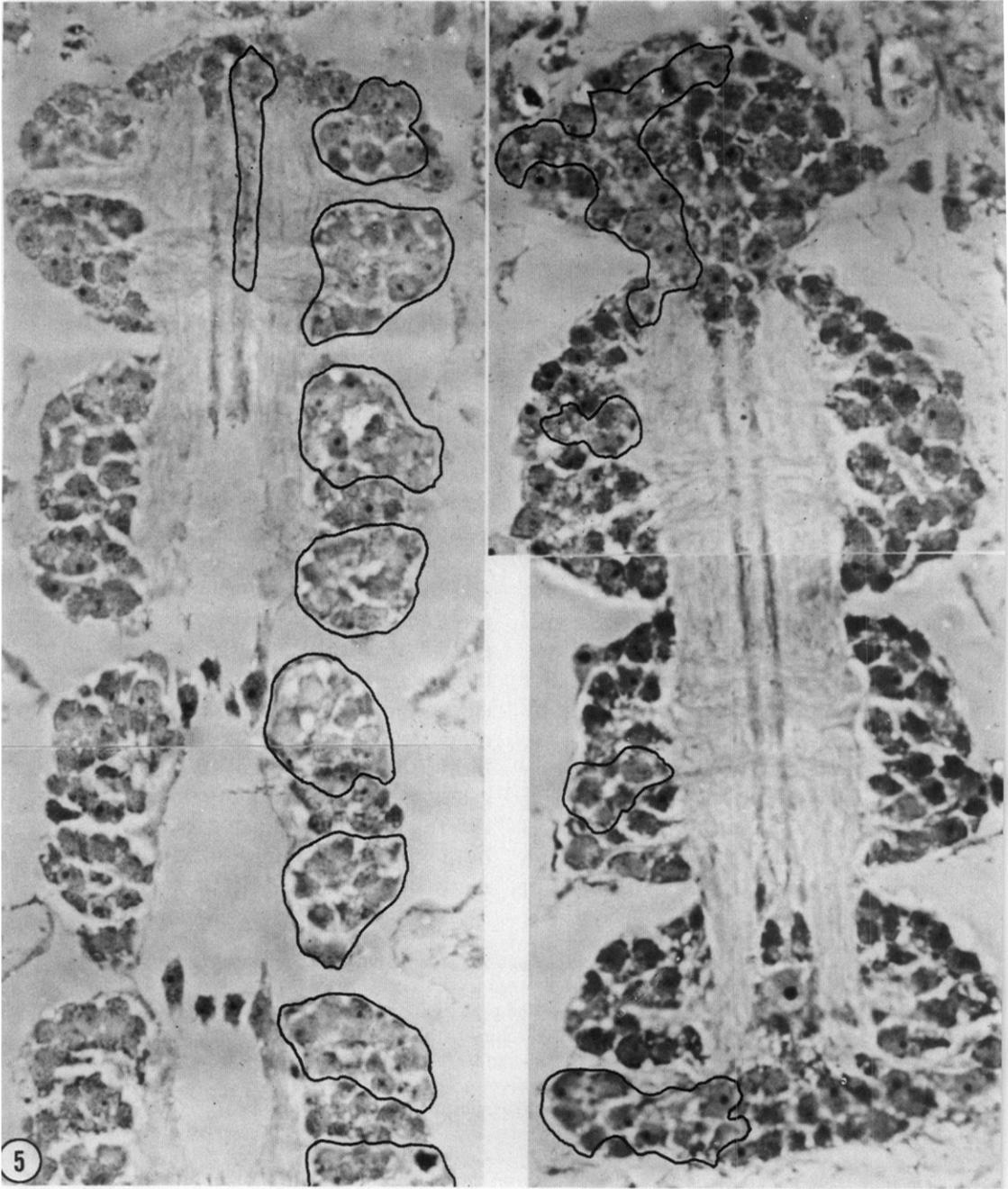


FIG. 5.

taken. In one set of such experiments, the OPQ cell, precursor of the O, P, and Q teloblasts, was injected at stage 6a and development was allowed to proceed for 6 more days before fixation and HRP staining of the embryo. As was to be expected on the basis of the traditional view that the O, P, and Q teloblasts give rise to nonnervous ectoderm (Whitman, 1887; Bergh, 1891; Bürger, 1902; Schleip, 1936), tissues other than the ventral nerve cord are found to be stained ipsilaterally to the HRP-injected OPQ cell (Fig. 2d). However, here there also appear some segmentally repeated patches of stain whose position near the ventral midline suggests that they are located within the ventral nerve cord.

Comparison of the wholemount preparations of Figs. 2c and d suggests these patches of stain are just those groups of cells in each segmental ganglion that fail to take on stain following injection of the ipsilateral N teloblast. This suggestion is confirmed by sections of OPQ cell-injected specimens (Fig. 5b), which show that the patches of stain correspond to groups of cell bodies located within the hemiganglia that fail to stain following injection of the N teloblast (Fig. 5a).

It can be concluded, therefore, that the N teloblast pair is not the exclusive precursor of the cells of the leech nerve cord and that some of these cells arise from one or

more of the teloblasts derived from the OPQ cell pair. Since wholemounts of embryos (not presented here), in which either cell OP or cell Q had been injected with HRP at stage 6b show medially located patches of stain similar to those seen in Fig. 2d, we conclude that the O and/or P, as well as the Q teloblast, contribute to the nerve cord.

The two other groups of cells of the central nervous system that fail to stain after HRP injection of the N teloblast, i.e., the supraesophageal ganglion (Fig. 2e) and the nerve tract cells, also fail to stain after HRP injection of the OPQ teloblast precursor. As for the supraesophageal ganglion, we next tested the possibility that it is derived from the eldest stem cells of the n bandlet conceivably produced prior to injection of an N teloblast at stage 6a. For this purpose, HRP was injected into the ectoteloblast precursor cell NOPQ of stage 5 embryos and subsequent development was allowed to proceed for 5 more days. Under this protocol, the N teloblast would certainly receive the tracer enzyme before it begins stem cell production. However, after injection of cell NOPQ the supraesophageal ganglion was still unstained (Fig. 6a). Thus if the supraesophageal ganglion is derived from the N teloblasts its failure to stain after HRP injection of an N teloblast is not attributable to the precocious presence of

FIG. 5. Sections of HRP-injected embryos. In this and the following two figures the sections were counterstained with toluidine blue and photographed under phase contrast optics. The outlines indicate areas where HRP stain is visible (in yellow against a blue background) in the original sections. All stained cell bodies lie ipsilateral to the injected cell. (a) Cell N was injected at stage 6a; fixation and staining occurred 6 days later. This is a slightly oblique horizontal section showing four segmental ganglia through which the section passes at progressively more dorsal levels, from anterior (top) to posterior. First ganglion from top: at this level most of the cell bodies visible in the hemiganglion are stained. Some unstained cells can be seen at the anterior edge of the hemiganglion and at the exit of the segmental nerve root. Second through fourth ganglion: at these more dorsal levels the majority of the cells are still stained, but a greater number of unstained cells are visible between the anterior and posterior stained areas. Third and fourth ganglion: at this most dorsal level four unstained nerve tract cells are visible at the anterior edge of the ganglion. (b) Cell OPQ was injected at stage 6a; fixation and staining occurred 6 days later. Tangential horizontal section showing four segmental ganglia. First ganglion from top: at this ventral level, stained neurons can be seen in the rostral, caudal, and lateral edges of the hemiganglion. Unstained neurons can be seen at the medial edge. Second and third ganglion: at this more dorsal level, stained neurons are seen where unstained neurons are visible in the second through fourth ganglion of panel (a). Fourth ganglion: at this intermediate level stained neurons are visible in the posterior half of the hemiganglion. Scale bar: 50 μ m.

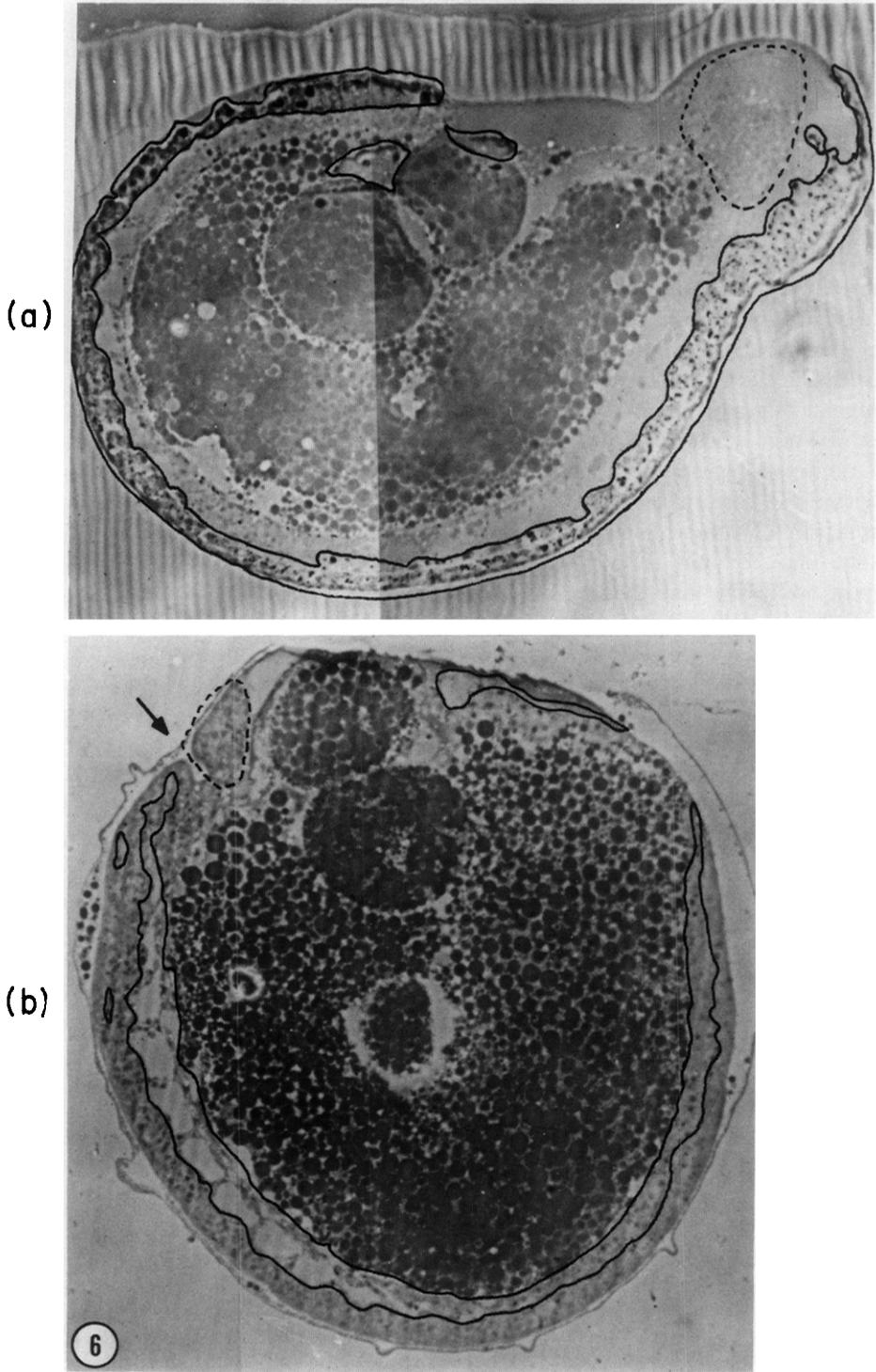


FIG. 6

n stem cells at the time of injection. The nerve tract cells also fail to stain following HRP injection of cell NOPQ (Fig. 7a). But almost all of the cells of the segmental hemiganglia ipsilateral to the injected NOPQ cell now stain (Fig. 7a). This finding is consistent with the previous conclusion that, between them, the N and Q teloblasts and the O and/or P teloblasts supply the neurons of the segmental ganglia. Nevertheless, in these specimens there are always a few (less than 10) unstained cell bodies visible in each hemiganglion. Although no positive identification of these exceptional cells has been made, it will be shown below that their failure to stain following injection of cell NOPQ is unlikely to be an experimental artifact.

Origin of the Mesoderm

In order to probe further into the origins of the hitherto unstained cells of the nerve cord, one of the pair of M mesoteloblasts of stage 4c *Helobdella* embryos was injected with HRP and subsequent development allowed to proceed for 6 more days. As was to be expected on the basis of the traditional view that the M teloblast pair is the source of the leech mesoderm (Whitman, 1878, 1887; Schleip, 1936), wholemounts of such preparations show extensive staining of germinal plate tissues outside the ventral nerve cord (Fig. 2f). Moreover, stain is also manifest beyond the lateral edge of the germinal plate, within the domain of the micromere cap. That stain appears in the form of circumferential fibers extending from the germinal plate to the dorsal midline. Thus the cells composing these fibers

seem to have been left behind by the m bandlets during the circumferential migration of the germinal bands at stage 8. In addition, horizontal sections of such preparations show that the nerve tract cells are now stained, as are a few cell bodies within the ganglion itself and thin cell profiles enveloping it (Fig. 7b). Although these stained intraganglionic cell bodies have not been positively identified, they are probably the same few cells that fail to stain after injection of cell NOPQ. Thus some cells of the leech nerve cord are evidently derived from mesoteloblast M.

In order to test the traditional view of the M teloblast as a source of mesoderm (Whitman, 1878, 1887) the developmental fate of the progeny of the stem cells of the m bandlet was examined further. For this purpose, cell DM of stage 4b embryos was injected with HRP and allowed to develop to completion of stage 8. Sections of such embryos show stained segmental blocks of tissue, corresponding to primordial somites, beneath the epithelial covering of the embryo (Fig. 6b). Hence the traditional assignment of the M teloblast to the role of mesodermal precursor cell is confirmed. By injecting the immediate precursor of the mesoteloblasts, we could be sure that the M teloblasts received HRP before any m stem cells had been produced; under such circumstances the supraesophageal ganglion still fails to stain (Fig. 6b).

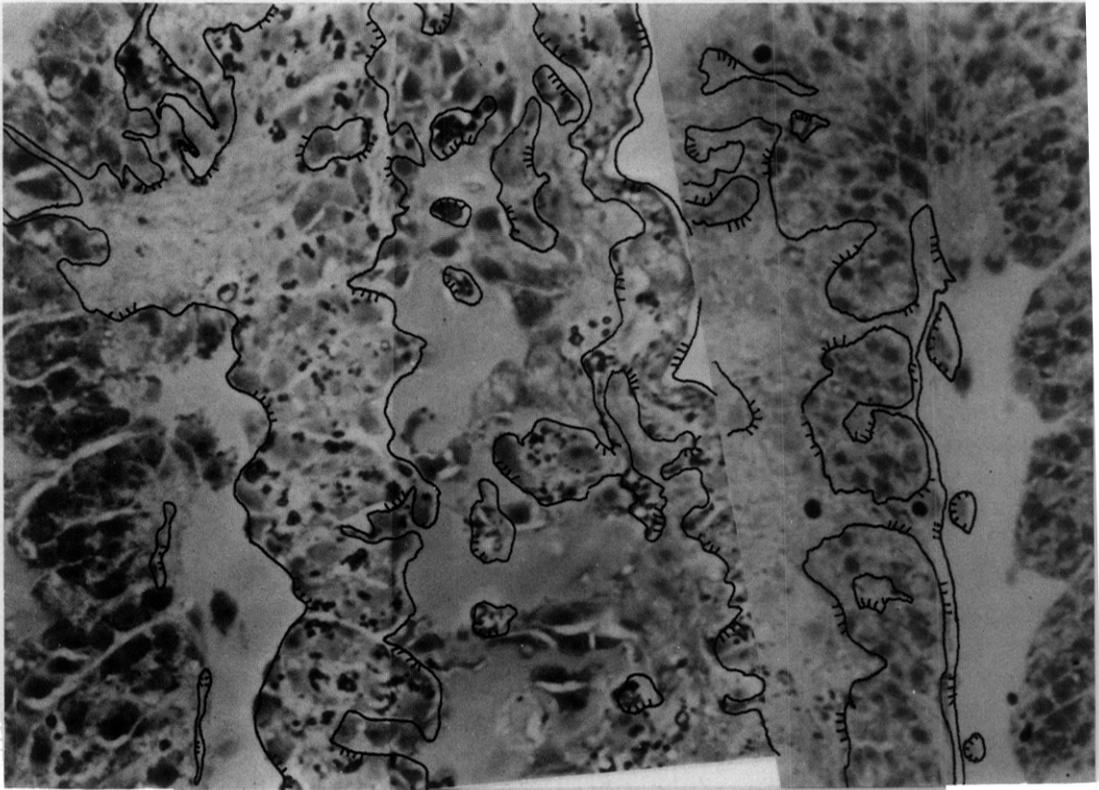
Origin of the Supraesophageal Ganglion

Provided that the failure of the supraesophageal ganglion to stain following injection of HRP into any of the teloblasts or

FIG. 6. Sections of HRP-injected embryos. (a) Cell NOPQ was injected at stage 5; fixation and staining occurred 5 days later. Sagittal section close to the midline, with the head lying to the right. Body wall and nerve cord are heavily stained, except for the head and the supraesophageal ganglion (marked by a dotted outline), which are unstained. The two large cell profiles near the top of the figure are teloblast remnants. The darkly counterstained granules filling the center of the section are yolk platelets from the macromeres and the teloblasts enclosed within the primordial gut. (b) Cell DM was injected at stage 4b; fixation and staining occurred at the completion of stage 8. Sagittal section, with future head lying at left. The segmentally iterated blocks of mesodermal tissue are stained. The head region (marked by a dotted outline) lying rostrally to (apparently above) the oral cavity (arrow) is unstained. Three teloblast remnants are visible within the primordial gut. Scale bar: 100 μ m.

(a)

(b)



(c)



FIG. 7.

teloblast precursors is not attributable to an artifactual cause, these results support Whitman's contention that the frontmost part of the leech nerve cord is derived from a source other than the germinal bands. A plausible alternative source is the micromere cap. To test this possibility HRP was injected into an A or B or C macromere of stage 3 embryos, i.e., prior to micromere formation. (Because of the small size of the micromeres the preferable procedure of directly injecting them in stage 4a *Helobdella* embryos is not feasible with our present techniques.) After 6 days of further development the embryos were fixed and stained. Wholemounds of such preparations show staining of only the frontmost end of the advanced embryo, as well as of the gut containing the remnants of the injected macromere (Fig. 2g). Sections show that the rostral stain is confined to the supraesophageal ganglion and the frontmost body wall (Fig. 7c). In the specimen shown, the stain is confined to a contiguous group of cells on one side bordering the midline. This suggests that the corresponding group of cells on the other side is derived from a different macromere and that these macromere-derived cells do not normally cross the midline. Thus, in accord with Whitman's original inference, the supraesophageal ganglion does have a different embryological origin from the remainder of the nerve cord, namely the A, B, or C macromere, and presumably therefore, the

micromere cap. (The possible contribution of the D macromere to the supraesophageal ganglion has not yet been determined.)

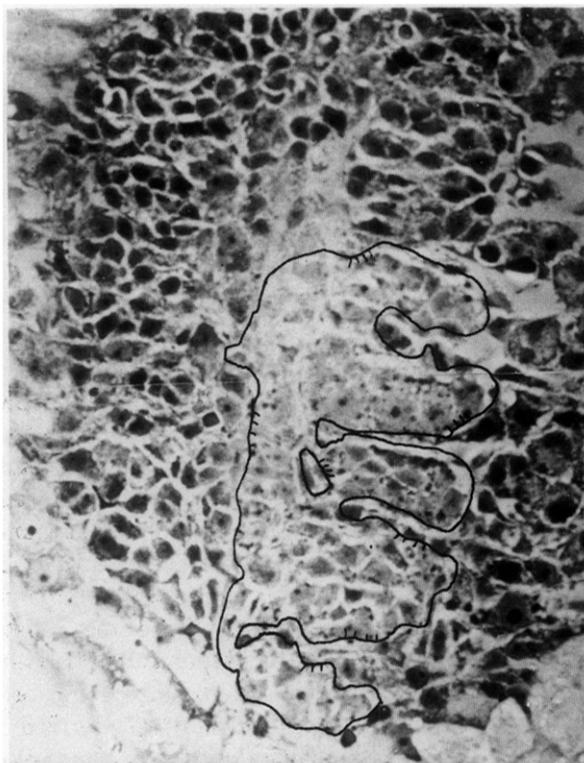
Number of Founder Cells of the Ganglion

Inasmuch as the segmental tissues and organs of the leech body arise from the germinal bands, it may be asked how many stem cells found any particular morphologically defined, segmental structure. In the case of the segmental ganglion it is already clear from the data presented here that the hemiganglion is founded by more than one stem cell. Since some of the neuronal cell bodies of the hemiganglion arise from stem cells of the n bandlet and others arise from stem cells of the q and o and/or p bandlets, it follows that at least three stem cells must contribute to the foundation of each hemiganglion. In addition, since some cells of the segmental ganglion are derived from the M teloblast, a fourth (m bandlet) stem cell must also contribute to the foundation of each hemiganglion. The following experiment was carried out to ascertain whether among the stem cells of just the n bandlet more than one contributes to the foundation of each hemiganglion.

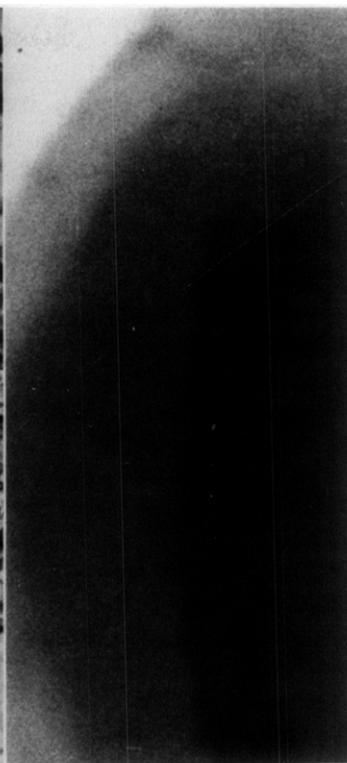
This experiment is made possible by the previously reported observation (Weisblat *et al.*, 1978) that if an N teloblast is injected with HRP only after some stem cells have been produced (for instance at stage 7), then, after further development the caudal, but not the rostral part of the ventral nerve

FIG. 7. Sections of HRP-injected embryos. In this and the following figure areas where HRP stain is visible in the original sections are indicated by a hachured outline, with the stain lying on the side of the hachure. (a) Cell NOPQ was injected at stage 5; fixation and staining occurred 5 days later. Horizontal section at a dorsal level, showing three segments. The hemiganglia and the ipsilateral body wall are heavily stained. Stained nerve cell processes extending across the midline are also visible in the ganglion at the top of the figure. The nerve tract cells are unstained. (b) Cell M was injected at stage 4c; fixation and staining occurred 6 days later. Horizontal section at a dorsal level showing four segments (with the anterior at the bottom). Extensive staining of tissue between nerve cord and body wall is seen. Essentially all of the neurons are unstained. Three stained and three unstained nerve tract cells can be seen. The sheet of connective tissue surrounding ganglionic cell packets is stained, as is one cell body within each of the two middle ganglia. (c) Cell A, B, or C was injected at stage 3 (the exact identity of the injected macromere, i.e., whether it was A or B or C, was not ascertained); fixation and staining occurred 6 days later. Oblique transverse section through the supraesophageal ganglion, shown dorsum up. Stained cells are visible next to the midline in the body wall of the head and supraesophageal ganglion. The esophageal tissue lying below the ganglion is not stained. Scale bar: (a), 40 μm ; (b) and (c), 60 μm .

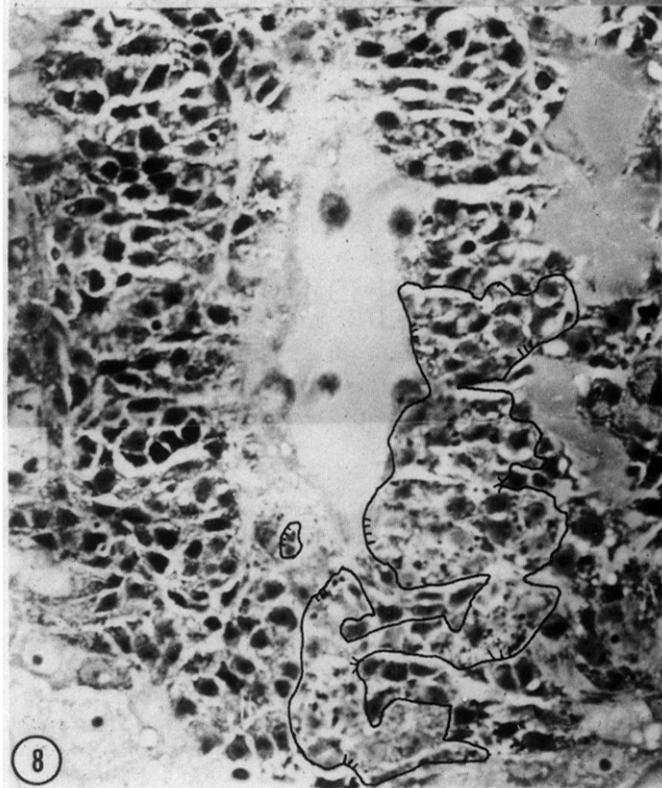
(a)



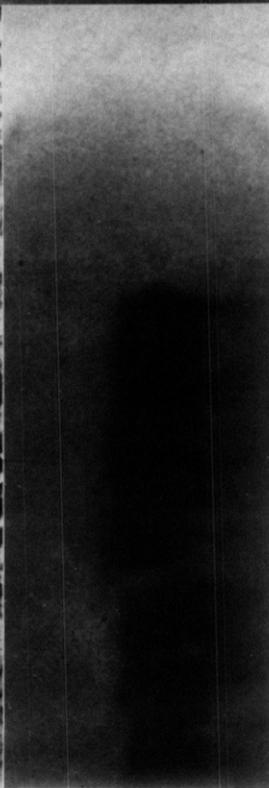
(b)



(c)



(d)



50 μm

100 μm

FIG. 8.
74

cord, stains. This finding showed that the caudal part of the hemilateral nervous system develops from the younger stem cells in the n bandlet. Moreover, the sharpness of the boundary between stained caudal and unstained rostral portions of the cord indicated that relatively little longitudinal migration, or mixing of elder and younger stem cell progeny, occurs in the germinal bands. Therefore, a detailed examination of the variation in position of the boundary between stained and unstained portions of the nerve cord should resolve the question of the number of n bandlet founder stem cells per hemiganglion. If the number of founder stem cells is not more than one, then the stain boundary should always be *interganglionic*, i.e., always fall between a fully stained posterior and a fully unstained anterior hemiganglion. But if that number is greater than one, then the stain boundary should often be *intraganglionic*, i.e., often fall within a hemiganglion, whose caudal part is stained and whose rostral part is unstained. In fact, the greater is the number of founder stem cells per hemiganglion, the higher would be the frequency of intraganglionic relative to interganglionic stain boundaries found in a set of embryos whose N teloblast was injected with HRP at stage 7.

We have found both inter- and intraganglionic stain boundaries in serial sections of different preparations (Figs. 8a and b). In a total of five sectioned preparations examined thus far, interganglionic boundaries were found in three cases and intraganglionic boundaries were found in two cases.

Inter- and intraganglionic stain boundaries are manifest also in wholemounts of such preparations (see Figs. 8c and d). In these embryos, which were fixed and stained at the time of hatching (and hence earlier in development than the embryo shown in Fig. 2c), the two transverse strips of stain in each ganglion are morphologically distinct, in that one extends further laterally than the other. Comparison of wholemount views and sections of such embryos shows that the laterally more and laterally less extensive strips correspond to groups of stained cells in the posterior and anterior parts of the hemiganglion, respectively. In some preparations the stain boundary falls rostral to one of the laterally less extensive transverse stained strips, in which case the boundary is interganglionic (Fig. 8c). And in other preparations the boundary falls rostral to one of the laterally more extensive transverse stained strips in which case the boundary is intraganglionic (Fig. 8d). We infer from the occurrence of some intraganglionic boundaries that the number of n bandlet stem cells that found each hemiganglion is greater than one. And since the chance of observing interganglionic boundaries decreases as the number of founder stem cells increases, it can be inferred from the relatively frequent occurrence of such boundaries that this number is unlikely to be much greater than two. In case it is two, it would appear that one (elder) n stem cell gives rise to neurons of the anterior lateral cell packet and of the ipsilateral half of the anterior ventromedial cell packet. The second (younger) n stem cell would give rise

FIG. 8. Inter- and intraganglionic stain boundaries. Each panel shows a different embryo in which cell N was injected with HRP at stage 7; fixation and staining occurred 3 days later [in (a) and (c)] or 4 days later [in (b) and (d)]. Anterior at the top. (a) and (c): Toluidine-blue counterstained horizontal sections showing three early segmental ganglia prior to their eventual longitudinal separation. In both (a) and (c) the first ganglion (from the top) does not and the third ganglion does contain stained cells. But in (a) the second ganglion contains stained cells in both anterior and posterior halves of its hemiganglion. By contrast, in (c) the second ganglion contains stained cells only in the posterior half of its hemiganglion. (b) and (d): Wholemount view of part of the embryonic nerve cord. In both (b) and (d) the caudal part of the nerve cord is stained and the rostral part is unstained. But in (b) the laterally less extensive transverse strip and in (d) the laterally more extensive strip is the first to be stained. In (a) and (b) the stain boundary is *interganglionic*, whereas in (c) and (d) it is *intraganglionic*.

to neurons of the posterior lateral cell packet and to the ipsilateral half of the posterior ventromedial packet. However, the present, rather limited data do not rule out the possibility that the ventromedial packets are actually founded by an additional n stem cell.

DISCUSSION

The results of the cell lineage analysis presented here confirm the previously held view that in glossiphoniid leeches the N and M teloblast pairs are precursors of nervous system and mesoderm, respectively. However, in contrast to the previously held view that the O, P, and Q teloblasts are mainly the source of circular muscles (Bergh, 1891; Bürger, 1902) we have found that a spatially coherent fraction of the neurons in each ganglion is derived from the OPQ teloblast precursor. Moreover, the positions within the ganglion of neuronal populations derived from each of these sources (N and OPQ) are relatively invariant from segment to segment (cf. Figs. 2c and d) and from specimen to specimen (cf. Figs. 8c and d). This finding suggests that the two cell groups of different developmental lineages might represent functionally distinct classes of neurons, in light of the relative positional invariance of functionally identified neurons in the segmental ganglia of *Hirudo medicinalis* (Nicholls and Van Essen, 1974) and *Haementaria ghilianii* (A. Kramer and J. Goldman, personal communication).

The HRP tracer method revealed, furthermore, that the cell bodies contributed by each teloblast are confined to the ipsilateral half of the embryonic ganglion. This finding suggests that the innervation of the contralateral musculature by motor neurons on the dorsal aspect of the adult segmental ganglion in *Hirudo* (Stuart, 1970) and *Haementaria* (Kramer and Goldman, personal communication) is attributable to a developmental decussation of their axons, rather than to an initially uncrossed projec-

tion followed by a reciprocal migration of the homologous cell bodies across the midline. In fact, in exceptionally well-stained preparations stained neuronal processes can be seen in the segmental nerves contralateral to the injected teloblast. [It should be noted that the finding of an exclusively ipsilateral cell origin applies only to *normal* embryonic development; following ablation of one N teloblast it is possible for the other N teloblast to make a contribution to contralateral hemiganglia (Blair and Weisblat, unpublished observations)]. Moreover, the present experiments show that each N teloblast contributes more than one, probably two, of its stem cells to the foundation of each ipsilateral hemiganglion.

The present experiments yield little information regarding the embryonic origin of the glial cells, since glia are not readily identifiable in even the most advanced embryos for which satisfactory HRP staining has been obtained. For instance the giant glia of the connective (cf. Figs. 4a and b) are not identifiable until about the 13th day of development. However, in transverse sections of N-cell injected embryos fixed and stained at the 7th day of development HRP-stain is seen in cells that appear to correspond to the nerve tract glial cells on the ventral aspect of the ganglionic neuropil (cf. Fig. 4c). This suggests that at least some of the glial cells are derived from the N ectoteloblast.

The mesoteloblast M was also found to contribute cells to the ventral nerve cord. Some of the M-derived cells within the segmental ganglion are likely to correspond to the muscle and sheath cells known to encapsulate the ganglion (of *H. medicinalis*) and its nerve cell packets (Coggeshall and Fawcett, 1964). Moreover, it is likely that the M-derived "nerve tract" cells are precursors of the muscle cells of the interganglionic connective since studies with another glossiphoniid leech, *Haementaria ghilianii*, have shown such a role for apparently homologous nerve tract cells (A.

Kramer, personal communication).

The finding that *only* frontmost body wall and the supraesophageal ganglion stain following HRP injection of a macromere is not in accord with the traditional view that the micromeres arising from cleavage of the macromeres are the main precursors of the micromere cap that covers the surface of the embryo behind the coalescing germinal bands during stage 8. Under that view it would have been expected that injection of a macromere would result in the presence of stain over large areas of the post-stage 8 embryo. The observed absence of stain can be explained in at least two different ways. In case the micromeres are mainly direct descendants of the macromeres, those micromeres that cover most of the embryonic surface might have undergone many more cycles of cell growth and division, and hence have diluted to a much greater extent any HRP passed on to them from their parent macromeres, than those micromeres that have remained near the site of their origin, i.e., the future head. Alternatively, it is possible that the micromere cap is a mosaic of cells of diverse lines of descent, of which only a minority, and in particular those cells that give rise to the supraesophageal ganglion, are lineal descendants of the A, B, and C macromeres. This latter possibility is supported by the finding (Fig. 2f) that stain appears within the domain referred to as "micromere cap" following injection of the mesoteloblast M.

The present demonstration that the supraesophageal ganglion is not derived from the germinal bands indicates that it is not to be included in the number of ganglia that arise through segmentation of the germinal plate. Since in the adult leech both rostral and caudal ganglia are fused and modified, it is not immediately obvious how many ganglionic primordia do arise through segmentation. However, detailed anatomical inspection of the adult subesophageal ganglion in the head and of the caudal tail ganglion at the rear indicates that the for-

mer corresponds to a fusion of 4 and the latter to a fusion of 7 ganglia, both in *Hirudo* and in glossiphoniid leeches (Harant and Grassé, 1959). Since the intervening, abdominal part of the ventral nerve cord consists of 21 separate ganglia, a total of 32 ganglia is to be accounted for by germinal plate segmentation. Direct observation of stage 8 glossiphoniid embryos confirms, furthermore, that 32 distinct ganglionic primordia arise in rostrocaudal progression during segmentation (Fernandez, 1980).

We thank Eduardo Macagno for communicating to us his unpublished method of preparing thick sections of Epon-embedded specimens, Kenneth Muller for helpful discussions, and Ellis N. Story for preparing the illustrations. This investigation was supported by NIH Postdoctoral Fellowship Grant NS05445, by NIH Research Grant NS12818, and by NSF Grant BNS77-19181.

Note added in proof. The present finding that the neurons of each ganglion of the leech nervous system are descended from several germinal bandlet cells resembles an earlier finding by Kankel and Hall (1976), based on the interpretation of tissue patterns of *Drosophila* mosaics, that the neurons of each ganglion of the fly nervous system are descended from three to ten blastoderm cells.

REFERENCES

- ANDERSON, D. T. (1973). "Embryology and Phylogeny in Annelids and Arthropods." Pergamon, Oxford.
- BERGH, R. S. (1891). Neue Beiträge zur Embryologie der Anneliden. II. Die Schichtenbildung im Keimstreifen der Hirudineen. *Z. Wiss. Zool.* **52**, 1-17.
- BÜRGER, O. (1902). Weitere Beiträge zur Entwicklungsgeschichte der Hirudineen. Zur Embryologie von Clepsine. *Z. Wiss. Zool.* **72**, 525-544.
- COGGESHALL, R. E., and FAWCETT, D. W. (1964). The fine structure of the central nervous system of the leech *Hirudo medicinalis*. *J. Neurophysiol.* **27**, 220-289.
- FERNANDEZ, J. (1980). Embryonic development of the Glossiphoniid leech *Theromyzon rude*: Characterization of developmental stages. *Develop. Biol.* **76**, 245-262.
- HARANT, H., and GRASSÉ, P. P. (1959). Classe des annélides achètes ou hirudinées ou sangsues. In "Traité de Zoologie" (P. P. Grassé, ed.), Vol. 5, pp. 509, 513. Masson, Paris.
- KANKEL, D. R., and HALL, J. C. (1976). Fate mapping of nervous system and other internal tissues in ge-

- netic mosaics of *Drosophila melanogaster*. *Develop. Biol.* **48**, 1-24.
- LUNA, L. G. (1968). "Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology," 3rd ed. McGraw-Hill, New York.
- MACAGNO, E. R. (1980). The number and distribution of neurons in leech segmental ganglia. *J. Comp. Neurol.*, in press.
- MANN, K. H. (1962). "Leeches (Hirudinea)." Pergamon, Oxford.
- MÜLLER, K. J. (1932). Über normale Entwicklung, inverse Asymmetrie und Doppelbildungen bei *Clepsine sexoculata*. *Z. Wiss. Zool.* **142**, 425-490.
- NICHOLLS, J. F., and VAN ESSEN, D. (1974). The nervous system of the leech. *Sci. Amer.* **230**, 38-48.
- SCHLEIP, W. (1936). Ontogenie der Hirudineen. In "Klassen und Ordnungen des Tierreichs" (H. G. Bronn, ed), Vol 4, Div. III, Book 4, Part 2, pp. 1-121. Akad. Verlagsgesellschaft, Leipzig.
- STUART, A. E. (1970). Physiological and morphological properties of motoneurons in the central nervous system of the leech. *J. Physiol. (London)* **209**, 627-646.
- WEISBLAT, D. A., SAWYER, R. T., and STENT, G. S. (1978). Cell lineage analysis by intracellular injection of a tracer. *Science* **202**, 1295-1298.
- WHITMAN, C. O. (1878). The embryology of *Clepsine*. *Quart. J. Microscop. Sci. (N. S.)* **18**, 215-315.
- WHITMAN, C. O. (1887). A contribution to the history of germ layers in *Clepsine*. *J. Morphol.* **1**, 105-182.